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ISOLATION AND CHARACTERISATION OF CRUDE OIL DEGRADING BACTERIA FROM CRUDE OIL CONTAMINATED AREAS

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author SPGL designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author VR managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The present study aimed to isolate, identify and characterize oil-degrading microbes from oil-contaminated sites. In this study, 26 bacterial strains were isolated from crude oil-contaminated sites and identified by using morphological, biochemical and molecular analysis by DNA gene sequencing. Their ability to biodegrade crude oil was determined by screening for bio-surfactant activity and by employing well diffusion assays. From these 26 strains, 3 bacterial isolates proved to be able to degrade all the 4 crude oil components (petrol, diesel, kerosene, engine oil). The isolates were subjected to morphological and biochemical characterisation, optimization for pH and temperature and PCR and DNA sequence analysis for identification. Based on biochemical and molecular characterization, these isolates were identified as *Psuedomonas aeruginosa, Enterobacter aerogenes* and *P. fluorescens*.

Keywords: Bioremediation; biosurfactant; oil degrading bacteria; *Pseudomonas aeruginosa; Enterobacter aerogenes; Pseudomonas fluorescens.*

ABBREVIATIONS

HDB :hydrocarbon degrading bacteria;
PCR :polymerase chain reaction;

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1. INTRODUCTION

In the present world anthropogenic activities such as growth, biological, physical, economic, industrial and infrastructure growth, science and technological growth etc revolve around energy. Apart from traditional sources of energy like fire woods, wind power, solar power etc., petroleum hydrocarbons continue to be used as the principle and adaptable form of energy. The most important strategic resource of any country is its crude petroleum resources [1]. In fact, all the human activities are counting on petrochemical industry to meet their energy needs. However, the use of this crude petroleum compounds seems to have a deteriorating effect on our environment [2].

Crude petroleum is a complex mixture of hydrocarbons, mostly saturated or aromatic whose molecular sizes are separated into fractions based on boiling points. The components are dissolved natural gas, gasoline, benzene, xylenes, naphthalenes, octanes, camphor, kerosene, diesel, fuel, heating oil and tars [3,4]. Petroleum hydrocarbon pollution is one of the most important pollution affecting the world these days. Even polar regions are not excluded from is harmful effects [5]. Sudden introduction of massive amounts of these xenobiotic chemicals in to the environment can affect the self cleaning capacity of the recipient ecosystem, hence results in the accumulation of these pollutants to a problematic levels.

Petroleum hydrocarbon pollution is one of the most important pollution forms affecting the world these days. The adverse effects of oil pollution on environment are well known [6]. For instance, the Deep Water Horizon oil spill accident in the Gulf of Mexico produced an intense and negative impact on the economy and environmental safety, which is still the focus of people's attention [2]. A number of marine organisms perish when such unfortunate events occur, and it destroys that natural environment for several years. Although people show increasing concern about the toxic effects of oil pollution on humans and animals in affected areas [7, 8] the strong toxic impacts of crude hydrocarbons on affected microbial communities are often ignored [9, 10]. Labud et al. [11] reported that petroleum hydrocarbons inhibited microbial biomass, and that the greatest adverse effects were observed in the gasoline-polluted sandy soil. In certain researches conducted based on diesel exposed areas, researchers found that the adverse effects of diesel fuel toxicity were reductions in species richness, evenness and phylogenetic diversity, which resulted in a community being heavily dominated by a few species, principally *Pseudomonas.*

Bioremediation in case of crude petroleum means microorganisms are employed in reducing the concentration or toxic nature of various chemical substances, such as petroleum derivatives, aliphatic and aromatic hydrocarbons, industrial solvents and pesticides etc. Not all microorganisms have the ability to degrade all the different compounds of crude petroleum. Environmental factors such as temperature, nutrients, electron acceptors and substrates have seemed to exhibit very important roles in bioremediation and influence biodegradation reactions [12]. This is why most researchers have found that many petroleum hydrocarbon-degrading bacteria showing excellent results during the degradation of petroleum compounds under laboratory conditions yet they exhibit dissatisfactory results in field-scale tests [13].

Most of the petroleum hydrocarbons produced in the environment are ultimately degraded by indigenous bacteria to meet their energy requirements to fulfill their growth and reproduction as well as the requirement to relieve physiological stress due to the presence of petroleum hydrocarbons in the microbial bulk environment [14,15]. Studies revealed that large number of petroleum degrading bacteria are found in oil spill contamination sites and oil reservoirs [14, 16]. Longer aged contamination areas showed increased number of microorganisms. And these abundance seems to show close relation with the types of petroleum hydrocarbons and the surrounding environmental factors [17,18].

Therefore, this paper provides an overview of the usage of bacteria as biodegraders. This study was undertaken to evaluate the capability of native bacterial strains to utilize the petroleum oils as the sole carbon source under *in vitro* conditions. In this study highly potent hydrocarbon degrading bacteria were isolated from two automobile workshops in Cherthala region in Kerala, India and were further identified using biochemical tests and molecular identification. Thus the aim of the present study was to investigate possible hydrocarbon degrading bacteria which help in the biodegradation of hydrocarbon in soil.

2. MATERIALS AND METHODS

2.1 Sampling and Initial Preparation

Oil contaminated soil and waste water samples were collected from oil contaminated areas of 2 different automobile workshops located in Cherthala region Alappuzha district, Kerala. Sample 1 - Soil sample (9°42'22''N76°19'02''E 382 m), Sample 2 - Soil sample (9°42'10'' N76°19'13''E504 m), Sample 3 -

Waste water sample - (9°42'22''N76°19'02''E 382 m). These samples were aseptically collected in sterile Polythene bags. The collected samples were labelled transferred to laboratory and stored at -4°C till further analysis.

2.2 Isolation of Bacteria from Sample

10 g of each soil sample was weighed and 10 ml of waste water sample measured and added to 3 separate conical flasks containing 90 mL of saline solution and stirred well and marked as sample 1, 2 and 3.

2.2.1 Culture medium and cultivation conditions

These 3 samples underwent serial dilution of different concentrations from 10^{-1} to 10^{-6}. These dilutions were made in 0.1 N saline, which was prepared by adding 2.55 g NaCl in 300 mL distilled water in 6 different test tubes. The sample obtained was serially diluted up to 10^{-6} dilution and this diluted culture was transferred to nutrient agar medium to perform spread plate technique. 0.1 mL of the diluted sample was inoculated with the glass spreader to the nutrient agar plates. These plates were then incubated for 24 hours at 37°C, after incubation numerous bacterial colonies were found on these agar plates and each colony was marked. Morphologically different specimens from the samples are then sub-cultured by streak plate method. Streak plating is a qualitative isolation method aimed to reduce the number of bacteria. Each bacterial colony are spread thinly over the plate in series of parallel lines, in different segments of the plate using a sterile loop and the plates were incubated for 24 hours at 37°C. Confluent growth of colonies was observed at the primary inoculation sites, but well isolated colonies were obtained over the final series of streaks.

2.3 Screening of Oil Degrading Bacteria

2.3.1 Screening of biosurfactant activity by blood agar analysis

Blood agar plates are prepared by mixing 5 mL of human blood to 100 ml nutrient agar plates and the bacterial isolates are streaked on to it and these plates are then incubated for 48 hours at 3°C. These plates were then observed for clearing zones (haemolysis) around the colonies, indicative of biosurfactant biosynthesis. Bacterial colonies around which haemolysis occurs were taken for further processes.

2.3.2 Screening of oil degrading activity by well diffusion assay

Bacterial colonies isolated after primary screening are then undergone secondary screening by well diffusion method. Well diffusion is performed with the available petroleum components such as petrol, diesel, kerosene and engine oil to identify bacteria with crude oil degrading potential. For well diffusion method nutrient agar plates with created wells are needed along with bacterial cultures inoculated nutrient broths.

(i) Well diffusion assay

To the nutrient agar plates, bacterial samples are inoculated by swabbing the surface with sterile cotton moistened with bacterial suspension and to the wells punched 100µl petroleum products are added and marked ' P' -petrol, 'D' -diesel, 'K' -kerosene and 'E' -engine oil and 'C' -control (distilled water). The plates were then incubated in upright position at 37°C for 24 hr. If the bacterial culture grew around the particular well that bacteria seems to have the property to degrade that particular crude oil compound.

2.4 Morphological and Biochemical Characterization

The morphological and biochemical characterization of crude oil degrading bacteria was done in accordance with the Bergey's manual of systematic bacteriology.

2.4.1 Gram staining

The gram stain reaction is based on the difference in the chemical composition of bacterial cell walls and is used to differentiate bacteria to 2 large groups Gram-positive and Gram-negative.

2.4.2 Biochemical tests

Identification of selected isolates were studied based on different biochemical characteristics like indole test, methyl red test, Voges-Proskauer test, citrate utilization test, triple sugar iron-agar test, mannitol-motility test, catalase test, oxidase test.

2.5 Optimization Parameters

The bacterial isolates with the highest activity were selected for optimization of oil degradation activity. Optimization parameters are used to check microbial growth rate at different temperature (30°C, 35°C, 37°C and 40°C) overnight and different pH (6, 6.5, 7.5, 8) incubated overnight.

2.6 Identification and Characterization of Isolates

DNA Isolation and PCR were performed. The PCR products were analysed using Standard DNA Marker (Commercially available 500 kb DNA ladder was

used as standard molecular weight DNA marker to determine the weight of amplified product) and purification and DNA sequencing of samples (amplified PCR products were purified, analysed and sequenced by automated DNA sequencing method) performed.

2.7 Database Similarity Search Using NCBI BLAST

Obtained sequences were used for database similarity search to find sequence match using nucleotide BLAST programme in NCBI Gen Bank (www.ncbi.nlm.nih.gov).

3. RESULTS

3.1 Isolation of Bacteria from Sample

3.1.1 Sub-culture

12 bacterial isolates of Sample 1, Sub cultured by Streak plate method and marked C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12. (Fig. 1).

6 bacterial isolates of Sample 2, Sub cultured by streak plate method and marked C1, C2, C3, C4, C5, C6. (Fig. 2).

8 bacterial isolates of Sample 3, Sub cultured by streak plate method and marked C1, C2, C3, C4, C5, C6, C7, C8. (Fig. 3).

3.2 Screening of Oil Degrading Bacteria

3.2.1 Screening of biosurfactant activity by blood agar analysis

From Sample 1, only 8 bacterial colonies showed haemolysis: C1, C5, C6, C8, C9, C10, C11, C12. (Fig. 4)

From Sample 2, No bacterial colonies showed haemolysis. (Fig. 5)

From Sample 3 only 2 bacterial colonies showed haemolysis: C2, C4. (Fig. 6)

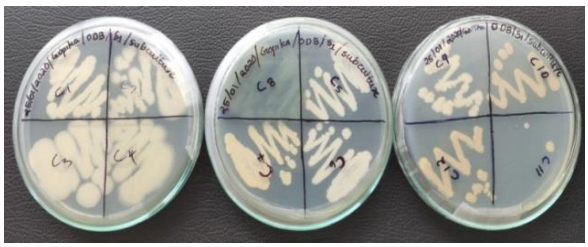

Fig. 1. Streak plate culture of bacterial colonies isolated from sample 1

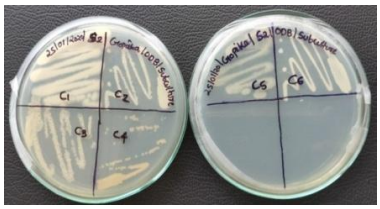

Fig. 2. Streak plate culture of bacterial colonies isolated from sample 2

Fig. 3. Streak plate culture of bacterial colonies isolated from sample 3

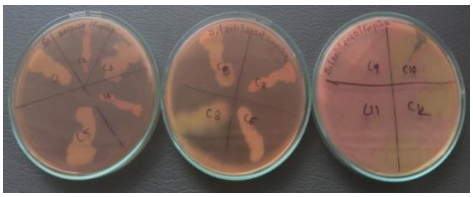

Fig. 4. Blood agar analysis of sample 1

Fig. 5. Blood agar analysis of sample 2

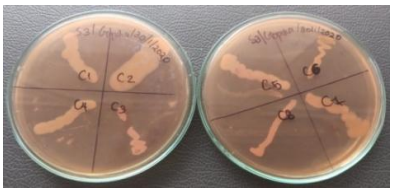

Fig. 6. Blood agar analysis of sample 3

After primary screening only selected bacterial colonies of sample 1 and 3 were used for secondary screening process.

3.2.2 Screening of oil degrading activity by well diffusion assay

- Hydrocarbon degrading bacteria grew around the well it is oil degrading bacterial strain
- Zone formation occurred around well – no potential for oil degradation.

Sample 1 - (Fig. 7), sample 3-(Fig. 8).

From the result of the screening tests performed above a total of 3 bacterial colonies of higher degrading property were selected as tolerant strains to all 4 of the available crude oil components tested. 3 Bacterial colonies proved to have high oil degrading property, they are: Sample 1 - C8, C12; Sample 3 - C4.

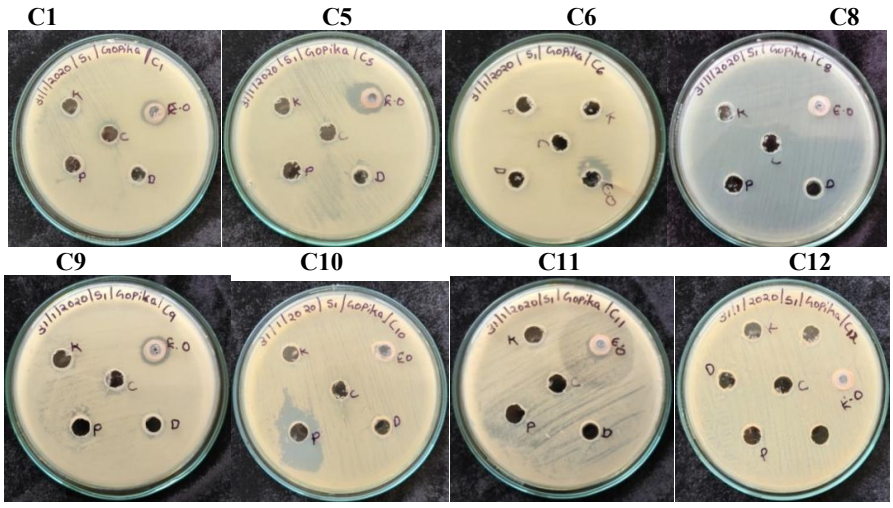

Fig. 7. Well diffusion assay-1 of selected sample 1

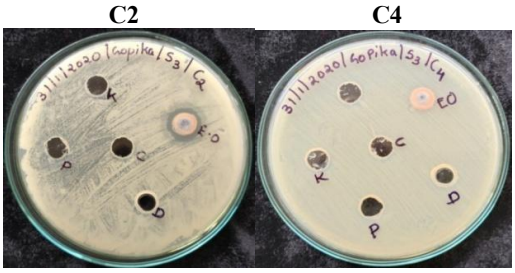

Fig. 8. Well diffusion assay-1 of selected sample 3

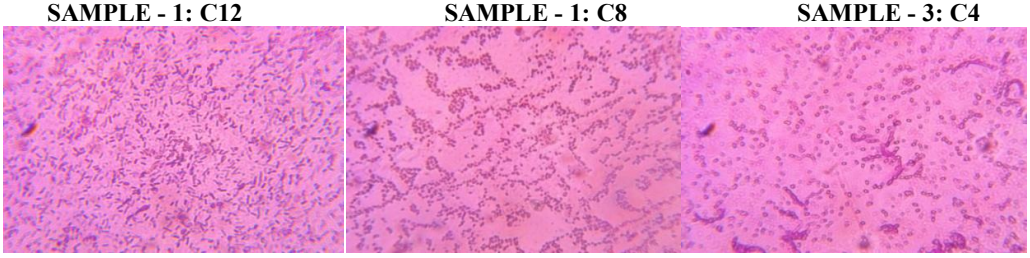

Fig. 9(a). Gram staining **Fig. 9(b). Gram staining** **Fig. 10. Gram staining**

Table 1. Gram staining and biochemical reactions

Test	Result		
	Sample 1: C8	**Sample 1: C12**	**Sample 3: C4**
Gram staining	Gram -ve	Gram-ve	Gram-ve
Indole test	-ve	-ve	-ve
Methyl red test	-ve	-ve	-ve
Voges proskauer test	-ve	+ve	-ve
Citrate test	+ve	+ve	-ve
Triple sugar iron agar [tsi] test	No carbohydrate production	No carbohydrate production but gas produced	No carbohydrate production
Mannitol motility test	Fermenting ,Motile	Fermenting , Motile	No Fermenting , Non Motile
Catalase test	+ve	+ve	+ve
Oxidase test	+ve	-ve	+ve

3.3 Morphological and Biochemical Characterization

3.3.1 Gram staining

Gram staining of the three selected samples showed 1 Gram negative cocci and 2 Gram negative rod shaped bacterial strains (Fig. 9 (a) – C8 & 9(b) – C12 of sample 1) (Fig. 10 – C4 of Sample 3).

3.3.2 Biochemical tests

The 3 selected isolates showed different biochemical results. (Table 1)

3.4 Optimization Parameters

3.4.1 Effect of pH

Maximum activity was seen in an increasing pH between 6-8. The optimum pH clearly marked at 6.5. And reduction in degradation seen at pH 8 and pH above 8. (Table 2).

Effect of temperature: Maximum activity seen in an increasing tempearature from 30-40°C, in soil environment, whereas 20-30°C in case of freshwater. But above 40°C marked a decreased degradation activity. (Table 3).

Table 2. Effect of pH

Bacterial strain	pH			
	6	6.5	7.5	8
Sample 1: c8	0.44	0.63	0.60	0.39
Sample1: c12	0.48	0.62	0.57	0.38
Sample1: c4	0.55	0.72	0.63	0.50

Maximum activity was seen in an increasing pH between 6-8. The optimum pH clearly marked at 6.5. And reduction in degradation seen at pH 8 and pH above 8

Table 3. Effect of temperature

Bacterial strain	Temperature			
	30°C	35°C	37°C	40°C
Sample 1: c8	0.49	0.57	0.62	0.60
Sample 1: c12	0.48	0.55	0.60	0.51
Sample 1: c4	0.72	0.68	0.58	0.44

Maximum activity seen in an increasing tempearature from 30-40°C, in soil environment, whereas 20-30°C in case of freshwater. But above 40°C marked a decreased degradation activity

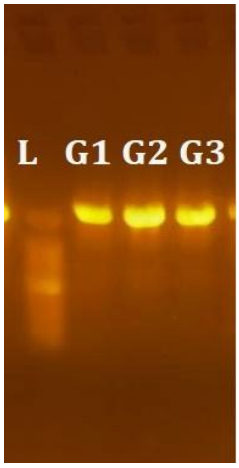

Fig. 11. DNA Isolation of selected bacterial isolates

Fig. 12. Amplified DNA using PCR: G1, G2, G3

3.5 Identification and Characterization of Isolates

DNA Isolation & Polymerase Chain Reaction: DNA isolation of selected bacterial strains (Fig. 11) were performed and they were further amplified using PCR (Fig. 12). After PCR these strains undergone sequence identification using NCBI BLAST.

3.6 Database Similarity Search Using NCBI BLAST

The sequences obtained were compared with the existing sequences in NCBI Gene bank through BLAST and the three bacterial strains were identified as:

Sample 1 - C8 / G1: *Pseudomonas aeruginosa* (96.97% homology was observed)
Sample 2 - C12 / G2: *Enterobacter aerogenes* (95.42% homology was observed)
Sample 3 – C4 / G3: *Pseudomonas fluorescens* (94.58% homology was observed)

4. DISCUSSION

Bioremediation acts on the whole system, the living soil communities and intents to restore the maximum health, diversity, and life. Today bioremediation is of prime impotance since it is regarded as the most effective and eco friendly means for removing crude oil pollution from contaminated sites, as this method makes use of microorganism. Microbial degradation of crude oil leads to production of organic acids and other metabolic products. However, their degradation products are not further analysed in this study. Indeed the study concentrates only on bioremediation efficiency.

The ability of the microorganisms to degrade the crude oil compounds was studied. And the study was based on the potential of isolated microorganisms to degrade available hydrocarbon products, such as petrol, diesel, kerosene and engine oil. For the study, samples collected from 2 different crude oil contaminated sites (2 automobile workshops) were taken. A total of 26 different bacterial colonies were isolated from the collected sample based on morphological differences such as pigmentation, consistency, form, margin of colony, elevation and shape of the colony. From the 26 bacterial isolates, 10 bacterial isolates were screened and recognized to have the ability to degrade different hydrocarbon products. Each bacterial colony seems to at least 2 or 3 of the above mentioned hydrocarbon products. From these 10 bacterial colonies, 3 bacterial isolates showed the ability to degrade all the four hydrocarbon products (petrol, diesel, kerosene and engine oil).

The selected hydrocarbon degrading bacterial strains (HDB) were subjected for Gram-reaction, Biochemical and Molecular identification. And the results were recorded after incubation along with the colony characteristics. The results of gram staining and biochemical tests were analysed on the basis of Bergey's manual of systematic bacteriology for identification of each bacterial isolate. The results were one gram –ve cocci bacteria and 2 gram –ve rod shaped bacteria. Optimization parameters based on temperature and pH were also noted, which further undergoes molecular characterisation including DNA isolation, PCR & Data base sequence analysis using NCBI blast. And finally these 3 most potential oil degrading bacterial starins identified as *Pseudomonas aeruginosa* (97.8), *Enterobacter aerogenes* (95.42%), *Pseudomonas fluorescens* (94.58%).

Among the many studies, in the literature regarding the use of different means to detect the biosurfactant activity from microorganisms. The lysis of red blood cells has been recommended as a preliminary test for Biosurfactant activity, and it serves as a simple and easy method encountering them in areas receiving petroleum waste discharges was not rare.

In a research conducted on samples collected from Tapis crude oil contaminated site of oil refinery at Kerteh, Terengganu, Malaysia [19]. The 11 bacterial isolates were successfully identified, and all of them showed potential in biodegrading petroleum hydrocarbon and producing extracellular biosurfactants. *Acinetobacter bauma*nii UKMP-12T, *Pseudomonas aeruginosa* UKMP-14T, *Rhodococcus sp.* UKMP-5T, and *Rhodococcus sp.* UKMP-7T were the most promising biosurfactant producers based on the entire method of assessment. From their previous study, *P. aeruginosa* strain isolated from two oil refineries in Malaysia (OGT-S1 and PPBST-1) was able to biodegrade the highest percentage of Sumandak oil (59%) and South Angsi oil (77%). Herein, *P. aeruginosa* UKMP-8T and *P. aeruginosa* UKMP-14T were able to grow in Tapis crude oil as the sole carbon source, and this suggested that the bacteria has the potential to biodegrade the hydrocarbons. In similarity to this research we also got the most potent bacteria as *P. aeruginosa*.

In a research conducted by Department of Microbiology, Rishi Biotech, Mumbai in samples collected from oil spill sites near seashore in Mumbai [20]. Four microorganisms were isolated by selective enrichment technique from petroleum contaminated-area. All four bacterial isolates were subjected for a preliminary assessment of their crude oil degradation efficiency. After 48 h of incubation, on nutrient plates overlaid with 100 µl of petroleum crude oil, the zone

of clearance were observed. Each bacterial isolate the average zone of clearance was observed and all the isolates proved to have maximum oil degradation ability. Each of the obtained hydrocarbon degrading bacterial strain (HDB) was subjected for the Gram-reaction, and colony, biochemical and physiological characteristics studies. The results of the biochemical test were recorded after incubation. The results of the biochemical tests performed analysed based on the Bergey's manual of systematic bacteriology for identification of each bacterial isolate. Out of the four bacterial isolates three were Gram negative while one isolate was Gram positive bacilli. Strains were identified as *Enterobacter* sp. and *Bacillus* sp., respectively. In comparison to this study our studies did used same processes but bacterial encounter were not same, we did not encounter *Enterobacter* or *Bacillus* sp., We did encounter 3 potent gram negative strains but none were gram positive. Among which one notably belonged to *Enterobacter sp*., which is *Enterobacter aerogenes.*

In a research conducted by MRD LIfeSciences Pvt. Ltd, Lucknow (U.P.) in samples collected from Pepper mill Colony, Nishatganj, Lucknow, Gomti nagar Petrol pump, Lucknow Unnao Petrol pump [21]. Three oil contaminated soil was taken and five cultures isolated and out of five cultures, only three cultures were identified and further used for experiment. The degradation of oil from culutre is check from three methods 1st is shake flask method 2nd is spread plate method and the last 3rd one is agar well diffusion method. There 3 cultures were isolated *Bacillus, Pseudomonas* and Staphylococcus they are showing positive results. The best oil degradation result found in engine and mobile-oil in the presence of *Pseudomonas fluroscence* culture. For checking the growth activity in bacterial culture. optimization technique was used and provide different carbon, nitrogen, suitable pH and optimum temperature as sources. shake flask method was again performed to check oil degradation activity of culture. In comparison to this study we encountered *Pseudomonas aeruginosa* as the most potent organism instead of *Pseudomonas fluroscence* [22, 23] And both these species were identified in the result.

Future prospects of the study involves in- situ and ex-situ remediation as well as phytoremediation. Bioremediation is an eco-friendly process which cleans the environment by using biological agents. The bacteria degrading crude oil can be of various types and the type of biosurfactant produced can also be variate. These biosurfactants can be isolated and their activities can be analysed using different types of substrates in the form of oil. Also varieties of indigenous hydrocarbon degrading bacteria from different contaminated sites can be studied on the basis of molecular analysis and produce an exclusively efficient consortium to treat different contaminated areas of both soil and water.

5. CONCLUSION

Hydrocarbon-degrading microorganisms exist in contaminated sites at a relatively high abundance level compared to non-contaminated soil. However, the degrader counts vary in different sites and probably vary in response to contaminant exposure time.

In future, we can go for the study of different petroleum contaminated sites and also study of different techniques which are used for removal of pollutants from soil and water environments by using the isolated microorganisms. We can also extend the study to exclusive bioremediation processes like study of isolated microorganisms on the basis of molecular characteristics and producing an effective consortium to treat petroleum contaminated sites. Apart from these the biosurfactant molecules produced by these hydrocarbon degrading bacteria can be isolated and it seems to be an important component with higher research potential. Further molecular studies are needed to decipher the catabolic genes resident in these isolates that were isolated from hydrocarbon polluted soils and their hydrocarbon specificities. These will invariably assist in developing cost effective and efficient bioremediation protocol for polluted soil. We can elaborate the study of enzymes which are responsible for the biodegradation process of petroleum hydrocarbons in the soil environment. The information obtained in this work will be the starting point for the design of molecular and chemical tools to further analyze the abundance and dynamics of this petroleum hydrocarbon degrading bacterial populations in the soil environments, and focusing on the microbial diversity under different environments. Reformulate this chapter: Conclusions should be a few short paragraphs summarizing the findings of your work and ending with potential further research directions on the subject.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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